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# Reporters of TCR signaling identify arthritogenic T cells in murine and human autoimmune arthritis

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**How pathogenic cluster of differentiation 4 (CD4) T cells in rheumatoid arthritis (RA) develop remains poorly understood. We used Nur77—a marker of T cell antigen receptor (TCR) signaling—to identify antigen-activated CD4 T cells in the SKG mouse model of autoimmune arthritis and in patients with RA. Using a fluorescent reporter of Nur77 expression in SKG mice, we found that higher levels of Nur77-eGFP in SKG CD4 T cells marked their autoreactivity, arthritogenic potential, and ability to more readily differentiate into interleukin-17 (IL-17)—producing cells. The T cells with increased autoreactivity, nonetheless had diminished ex vivo inducible TCR signaling, perhaps reflective of adaptive inhibitory mechanisms induced by chronic autoantigen exposure in vivo. The enhanced autoreactivity was associated with up-regulation of IL-6 cytokine signaling machinery, which might be attributable, in part, to a reduced amount of expression of suppressor of cytokine signaling 3 (SOCS3)—a key negative regulator of IL-6 signaling. As a result, the more autoreactive GFP<sup>hi</sup> CD4 T cells from SKGNur mice were hyperresponsive to IL-6 receptor signaling. Consistent with findings from SKGNur mice, SOCS3 expression was similarly down-regulated in RA synovium. This suggests that despite impaired TCR signaling, autoreactive T cells exposed to chronic antigen stimulation exhibit heightened sensitivity to IL-6, which contributes to the arthritogenicity in SKG mice, and perhaps in patients with RA.**

antigen receptor signaling | T cells | Nur77 | autoimmunity | rheumatoid arthritis

**R**heumatoid arthritis (RA) is a chronic, destructive autoimmune disease that targets both joints and other organs. Cluster of differentiation 4 (CD4) T cells have long been appreciated as playing a crucial role in the pathogenesis of RA (1–4). Cellular and biochemical analyses of human CD4 T cells have revealed abnormal T cell antigen receptor (TCR) signaling in RA patients (5–8). Surprisingly, CD4 T cells from patients with RA are hyporesponsive to TCR engagement ex vivo, as evidenced by reduced calcium mobilization and interleukin-2 (IL-2) production (9, 10). This may be due, in part, to reduced TCR $\zeta$  and linker of activated T cells (LAT) expression, as well as cellular changes associated with immune senescence (6, 7, 11–16). Although CD4 T cells from patients with RA seem to have decreased signaling capacity when stimulated in vitro, they appear to hyperproliferate during clonal expansion and differentiate into effector cells that drive disease (17, 18). It is not currently understood how to reconcile these paradoxical observations of diminished TCR signaling in the setting of increased T cell proliferation and effector functions, and it is not clear whether this RA T cell phenotype is directly causal in disease pathogenesis or rather results from exposure to chronic inflammation. The inability to identify relevant arthritogenic T cells in patients and in murine disease models has limited our understanding of disease-initiating events in RA. In this report, we have developed a strategy to overcome this limitation by taking advantage of the dynamic expression pattern of Nur77(*Nr4a1*) in T cells.

*Nr4a1* is an immediate early gene that encodes the orphan nuclear hormone receptor Nur77. It is rapidly and robustly up-regulated by TCR, but not cytokine, stimulation (19, 20). Therefore, Nur77 expression in murine and human T cells serves as a specific marker of TCR signaling but is insensitive to cytokine stimulation (21–24). Detection of Nur77 expression can be used to identify T cells stimulated by direct self-antigen exposure prior to disease initiation as well as in the context of complex immune responses and chronic autoimmune diseases in which inflammatory cytokines can influence T cell phenotypes. Indeed, gene expression data show that endogenous *Nr4a1* transcripts are highly up-regulated in autoantigen-specific CD4 T cells in vivo in the context of bona fide autoimmune disease (Immunological Genome Project; <http://www.immgen.org/>; ref. 25). This suggested to us that Nur77 expression could be employed to identify autoantigen-specific CD4 T cells in RA.

SKG mice harbor a profoundly hypomorphic variant of the Zap70 cytoplasmic tyrosine kinase, a molecule that is critical for proximal TCR signal transduction. As a consequence, SKG mice exhibit impaired thymocyte negative selection, giving rise to T cells with a more potentially autoreactive TCR repertoire (26). In response to either an innate immune stimulus in the form of zymosan injection or adoptive transfer of CD4 T cells into

## Significance

**How arthritis-causing T cells trigger rheumatoid arthritis (RA) is not understood since it is difficult to differentiate T cells activated by inflammation in arthritic joints from those activated through their T cell antigen receptor (TCR) by self-antigens. We developed a model to identify and study antigen-specific T cell responses in arthritis. Nur77—a specific marker of TCR signaling—was used to identify antigen-activated T cells in the SKG arthritis model and in patients with RA. Nur77 could distinguish highly arthritogenic and autoreactive T cells in SKG mice. The enhanced autoreactivity was associated with increased interleukin-6 (IL-6) receptor signaling, likely contributing to their arthritogenicity. These data highlight a functional correlate between Nur77 expression, arthritogenic T cell populations, and heightened IL-6 sensitivity in SKG mice with translatable implications for human RA.**

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The authors declare no conflict of interest.

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lymphopenic hosts, tolerance is broken and mice develop an erosive inflammatory arthritis that resembles RA. For example, rheumatoid factor, anti-cyclic citrullinated peptide (CCP) antibodies, and interstitial lung inflammation develop at the onset of disease (26). The SKG mouse provides a useful model to study early events in RA pathogenesis; not only does it capture many important features of human RA but SKG also offers 2 advantages over other RA models. First, unlike the more commonly used collagen-induced arthritis (27) or K/BxN serum-transfer models of arthritis, which both bypass the initial break in TCR tolerance (28), the SKG mice exhibit a loss in central, and likely peripheral, tolerance that can be molecularly dissected. Hence, this model uniquely allows us to study arthritis-causing T cells both before and during disease. Second, it recapitulates the paradoxical ability of RA CD4 T cells to differentiate into pathogenic effector cells despite impaired TCR signaling (6, 7, 11–18, 26).

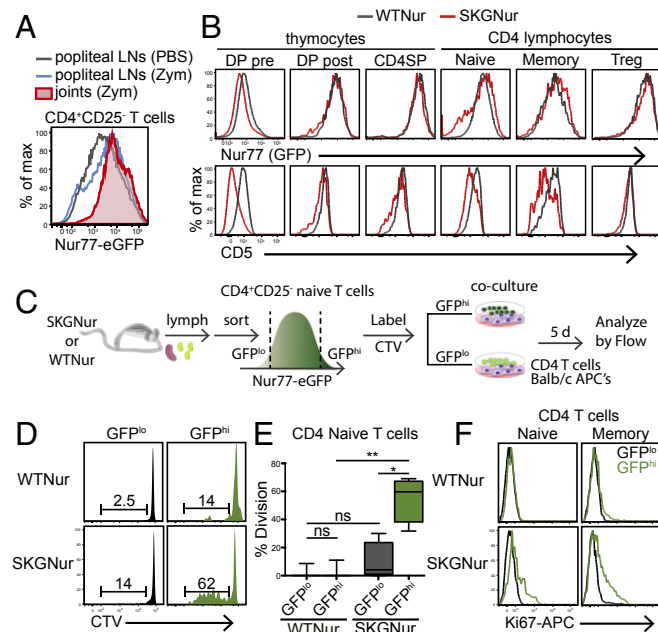
A recent study of the SKG model has identified 1 specific arthritogenic TCR directed against a ubiquitous self-antigen (29), but it is not known whether rare antigen-specific T cell clones drive disease or whether the entire preimmune TCR repertoire has arthritogenic potential because it is highly autoreactive. It is also not clear how tolerance of such clones is broken in the face of profoundly depressed TCR signaling in SKG mice. To address these questions, we backcrossed the Nur77-eGFP bacterial artificial chromosome (BAC) transgenic (Tg) reporter line [in which eGFP protein expression is under the control of the *Nr4a1* regulatory region (24)] onto the SKG mouse model of arthritis. The resulting so-called SKGNur mice provided us with a tool to facilitate identification and study of arthritogenic CD4 T cells both before and after disease initiation. Despite impaired TCR signal transduction, the peripheral naive CD4 T cells from SKG mice expressed higher levels of Nur77-eGFP relative to those of wild-type (WT) mice. This suggested that even before disease initiation, SKG mice harbor a profoundly autoreactive T cell repertoire that exhibits increased downstream signaling despite impaired Zap70 function. We show here that the amount of Nur77-eGFP expression in CD4 T cells from SKG mice correlated with both their autoreactivity and their arthritogenicity. We found that the SKGNur CD4 T cells that expressed the highest level of the Nur77-eGFP reporter ( $\text{GFP}^{\text{hi}}$ ) differentiated more readily into IL-17-producing cells in vivo. While examining why  $\text{GFP}^{\text{hi}}$  cells were predisposed to differentiate into T helper 17 (Th17) effector T cells, we observed profoundly enhanced IL-6-dependent signaling in  $\text{GFP}^{\text{hi}}$  CD4 T cells from SKG mice. Apparently, the autoimmune repertoire, despite being coupled with impaired TCR signal transduction capacity in the SKG model, resulted in reduced expression of suppressor of cytokine signaling 3 (SOCS3), a key negative regulator of IL-6 signaling. Likewise, we found SOCS3 expression was down-regulated in RA synovium. This, in turn, suggests that despite impaired TCR signaling, autoreactive T cells exposed to chronic antigen stimulation exhibit heightened sensitivity to IL-6 receptor (IL-6R) signaling, which contributes to their arthritogenicity in SKG mice, and perhaps in patients with RA.

## Results

**A Fluorescent Reporter of TCR Signaling Reveals and Marks Self-Reactivity of the CD4 T Cell Repertoire in SKG Mice.** We sought to take advantage of a transcriptional reporter of *Nr4a1* expression in order to examine antigen-specific signaling in pathogenic SKG T cells. Since Nur77 expression is induced downstream of TCR, but not cytokine receptor, signaling (21, 23, 24), it can serve as a specific reporter of antigen receptor signaling in T cells in vitro or in vivo (21–24). We backcrossed the Nur77-eGFP BAC Tg reporter (24) into either WT or SKG mice, both on the BALB/c background (hereafter referred to as WT Nur or SKGNur, respectively). We confirmed that the introduction of the Nur77-eGFP BAC Tg reporter into the SKG mouse model did not alter the phenotype. Indeed, the SKGNur CD4 T cells continued to demonstrate dramatic hyporesponsiveness to in vitro polyclonal

TCR stimulation, consistent with published data (26, 30), and developed a severe inflammatory and erosive arthritis after zymosan challenge (*SI Appendix*, Fig. S1 A–C).

It has previously been shown that thymic negative selection is quite impaired in SKG mice because TCR signaling is exceptionally dampened (26). As a result, highly autoreactive T cells, which would ordinarily be deleted, are instead positively selected. Although 1 SKG autoreactive TCR has been cloned (29), it remains unknown whether rare antigen-specific T cell clones drive disease or whether the entire repertoire has arthritogenic potential because it is highly autoreactive. The Nur77-eGFP distribution in SKG mice is broad and overlaps with the distribution seen in normal mice (Fig. 1 A and B). Following stimulation with zymosan, which induces arthritis, there is an enrichment of Nur77-eGFP-expressing CD4 T cells with higher levels of GFP in draining popliteal nodes, but the distribution of GFP is still quite broad and largely overlaps with that of cells from untreated SKG mice. The Nur77-eGFP expression in CD4 T cells isolated from the arthritic joints of SKG mice, however, is substantially increased but still overlaps with the expression distribution in CD4 T cells from untreated mice (Fig. 1A and gating in *SI Appendix*,



**Fig. 1.** Nur77 reporter of TCR signaling marks the selection of an autoreactive repertoire during thymic development with further pruning in the periphery. (A) Assessment of Nur77-eGFP fluorescence in SKGNur CD4<sup>+</sup>CD25<sup>−</sup> T cells freshly isolated from popliteal lymph nodes (LNs) ± phosphate-buffered saline (PBS) or zymosan (Zym) and arthritic joints (+Zym). Data are representative of 10 mice in 4 independent experiments. (B) Comparison of expression of Nur77-eGFP and CD5 in preselection (pre; CD69<sup>hi</sup>TCR-β<sup>lo</sup>) and postselection (post; CD69<sup>hi</sup>TCR-β<sup>hi</sup>) double-positive (DP), CD4SP (CD4<sup>+</sup>CD3<sup>hi</sup>TCRβ<sup>hi</sup>) thymocytes, and peripheral CD4<sup>+</sup>CD25<sup>−</sup> naive (CD62L<sup>hi</sup>CD44<sup>lo</sup>) and memory (CD44<sup>hi</sup>CD62L<sup>lo</sup>) and CD4<sup>+</sup>CD25<sup>+</sup> Treg lymphocytes. Results are representative of 2 independent experiments with 6 mice in each group. (C) Depiction of AMLR workflow. (D and E) Sorted CD4<sup>+</sup>CD25<sup>−</sup> naive (CD62L<sup>hi</sup>CD44<sup>lo</sup>)  $\text{GFP}^{\text{lo}}$  and  $\text{GFP}^{\text{hi}}$  T cells were loaded with CTV dye and cocultured with nonirradiated APCs from BALB/c mice for 5 d. (D) Cells were subsequently stained for T-cell markers and assessed for dye dilution by flow cytometry. \* $P < 0.05$ ; \*\* $P < 0.01$ . ns, not significant. (E) Box plots represent percent (%) division of  $\text{GFP}^{\text{lo}}$  and  $\text{GFP}^{\text{hi}}$  CD4 T cells as assessed using the Flow Jo v9 proliferation platform (center line, median; box limits, upper and lower quartiles; whiskers, 1.5× interquartile range). Representative histograms of CTV dilution (D) and % cell division (E) from 4 experimental replicates (each containing 2 to 3 pooled mice) are shown. (F) Histograms represent Ki67 proliferation marker staining in  $\text{GFP}^{\text{lo}}$  and  $\text{GFP}^{\text{hi}}$  CD4<sup>+</sup>CD25<sup>−</sup> naive (CD62L<sup>hi</sup>CD44<sup>lo</sup>) or memory (CD44<sup>hi</sup>CD62L<sup>lo</sup>) T cells. In C and E, a 2-tailed Student's *t* test was used.



Fig. S1D). This suggests that the T cell repertoire prior to and after zymosan stimulation has a broad distribution but that the arthritogenic potential may not be limited to a narrow spectrum of autoreactive cells. This could account for the failure to identify a unique dominant arthritogenic TCR in this model to date (29). We took advantage of SKGNur mice to study this further.

We first used the Nur77-eGFP reporter to assess differences in TCR signaling between WT and SKG T cells during T cell development, as it seemed likely that a more autoreactive repertoire was being positively selected by the self-peptide major histocompatibility complex (pMHC) in SKG mice. The surface marker CD5 is dependent on p56<sup>lck</sup> activity throughout T cell development and is frequently used as an indicator of TCR signaling strength during and after thymic development (31). We assessed both CD5 and Nur77-eGFP expression at sequential stages of T cell development in WTNur and SKGNur mice. Consistent with impaired TCR signaling in SKG mice as identified in previous studies, preselection double-positive thymocytes from SKGNur mice exhibited lower levels of both GFP and CD5 relative to those of WTNur (Fig. 1B and SI Appendix, Fig. S2A). Prior work has identified a minimal threshold of TCR signaling that is necessary for positive selection (32). Consistent with this, after the positive selection checkpoint, GFP and CD5 were markedly up-regulated in postselection thymocytes in both WTNur and SKGNur mice. Of note, GFP expression levels in SKGNur CD4 single-positive (CD4SP) thymocytes became equivalent to WTNur, whereas CD5 expression remained reduced in SKGNur CD4SP thymocytes (Fig. 1B and SI Appendix, Fig. S2A). Since TCR signaling is markedly dampened in SKG mice, high GFP expression in SKGNur CD4SP thymocytes implies selection of a highly autoreactive repertoire where strong stimulation by self-pMHC complexes compensates for impaired signal transduction in order to support positive selection (32).

Surprisingly, we found that some peripheral naive and memory CD4 lymphocytes from SKGNur mice had somewhat higher GFP levels than WTNur controls (Fig. 1B and SI Appendix, Fig. S2A). Note that regulatory T cells (Tregs), which are selected on the basis of self-reactivity during development and consequently express high levels of Nur77 (23) (Fig. 1B), were excluded in this analysis. This suggested to us that autoreactive SKG T cells were either exposed to chronic autoantigen stimulation in the periphery or underwent further repertoire pruning under the influence of autoantigens and weak TCR signaling, or both. In contrast, CD5 surface expression remained lower in SKGNur peripheral CD4 T cells compared with controls (Fig. 1B and SI Appendix, Fig. S2A). It is unclear why CD5 and Nur77 expression levels were discordant following positive selection and in the periphery. However, CD5 is frequently used as a marker of proximal TCR signaling strength involving interactions with Lck and Cbl with the CD5 cytoplasmic tail (9, 33, 34). In contrast, Nur77 is reflective of transcriptional activation that is regulated by tonic and integrated downstream TCR signaling pathways (35). Thus, we used Nur77-eGFP as a marker of TCR signaling in SKG peripheral T cells as it appeared to be a reliable marker of not only strength of transduced TCR signal but integrated TCR signaling reflective of antigen encounter in vivo over time.

**Nur77-eGFP High-Expressing CD4 T Cells Are Phenotypically Different from GFP Low-Expressing T Cells from WTNur and SKGNur Mice.** Given the broad distribution of Nur77-eGFP expression, we asked whether the extreme ends of the histogram represented phenotypic differences or differences in T cell subsets. To examine this, we compared the surface expression of T cell activation markers in the 10% highest and 10% lowest Nur77-eGFP-expressing subsets (hereafter referred to as GFP<sup>hi</sup> and GFP<sup>lo</sup>, respectively) from the total CD4 T cell population of WTNur and SKGNur mice. We found the GFP<sup>hi</sup> CD4 T cells expressed the highest levels of CD5, CD25, and CD69 from both strains of mice (SI Appendix, Fig. S3A). To determine the percentage of memory (CD44<sup>hi</sup>CD62L<sup>lo</sup>) and naive (CD62L<sup>lo</sup>CD44<sup>hi</sup>) T cell subsets in the total CD4 T cells and GFP<sup>hi</sup> and GFP<sup>lo</sup> populations, CD25<sup>+</sup>

T cells were gated out to exclude Tregs. SKGNur mice had reduced naive and expanded memory T cells compared with WTNur mice (SI Appendix, Fig. S3B and C). Although there were some differences in naive and memory subsets between GFP<sup>hi</sup> and GFP<sup>lo</sup> populations from WTNur mice, the largest differences were observed between GFP<sup>hi</sup> and GFP<sup>lo</sup> populations from SKGNur mice, with a significantly lower percentage of naive and a higher percentage of memory T cells in the GFP<sup>hi</sup> population (SI Appendix, Fig. S3B and C). Since CD25<sup>+</sup> T cells were primarily in the GFP<sup>hi</sup> population, we sought to determine how well this reflected the FoxP3<sup>+</sup> Treg population. GFP<sup>hi</sup> CD4 T cells from both WTNur and SKGNur mice were significantly enriched for FoxP3<sup>+</sup> Tregs and were expressed in a significantly higher percentage in both subsets from SKGNur mice compared with WTNur controls (SI Appendix, Fig. S3D–F).

**Nur77-eGFP High-Expressing SKGNur CD4 T Cells Demonstrate Increased Autoreactivity.** In view of the discordant expression of Nur77-eGFP and CD5 in peripheral CD4 T cells from SKGNur mice and the broad distribution of Nur77-eGFP expression, we sought to take an independent approach to probe the putative self-reactivity of the mature repertoire and whether Nur77-eGFP expression marked differences in the self-reactivity of this repertoire. We compared the response of CD4<sup>+</sup>CD25<sup>−</sup> naive T cells sorted for the 10% highest and 10% lowest Nur77-eGFP expression to determine whether reporter expression reflected repertoire differences and/or interactions with self-pMHC among the polyclonal CD4 T cells. Sorted CD4<sup>+</sup>CD25<sup>−</sup> GFP<sup>hi</sup> and GFP<sup>lo</sup> naive T cells from SKGNur and WTNur mice were challenged in an in vitro autologous mixed lymphocyte reaction (AMLR). Sorted cells were stained with Cell Trace Violet (CTV) and incubated in vitro with WT BALB/c splenocytes for 5 d in culture, and proliferation was assessed by CTV dye dilution (Fig. 1C). WTNur T cells are not expected to mount a response in this assay because of efficient tolerance induction during thymic negative selection. Indeed, as expected, neither GFP<sup>hi</sup> nor GFP<sup>lo</sup> CD4 T cells from WTNur mice mounted substantial proliferative responses. However, SKGNur GFP<sup>hi</sup> CD4<sup>+</sup>CD25<sup>−</sup> naive T cells exhibited at least a 4-fold greater in vitro proliferative response to autologous BALB/c antigen-presenting cells (APCs) compared with SKG-GFP<sup>lo</sup> CD4 T cells (Fig. 1D and E). The ability of SKG T cells to proliferate in an autologous AMLR has previously been shown to be MHC class II-dependent (36) and suggests that SKGNur GFP<sup>hi</sup> CD4 T cells are indeed enriched for cells with self-reactive TCRs, and that the degree of autoreactivity is sufficient to overcome impaired TCR signal transduction conferred by the SKG *Zap70* hypomorphic allele.

We then assessed GFP<sup>hi</sup> and GFP<sup>lo</sup> CD4 T cell populations from WTNur and SKGNur mice for evidence of an in vivo correlate of the in vitro proliferative response. As shown in Fig. 1F, we compared Ki-67 expression in both naive and memory CD4 T cells from WTNur and SKGNur mice. As expected, under steady-state conditions, virtually no naive WT T cells were proliferating, and Ki67 expression was virtually undetectable in both GFP<sup>hi</sup> and GFP<sup>lo</sup> CD4 T cells from WTNur mice. However, a substantial proportion of naive and memory T cells from SKG mice were Ki-67<sup>+</sup>, consistent with a previous 5-bromodeoxyuridine labeling study in which SKG CD4 T cells were shown to have high in vivo proliferative activity (36). Importantly, the proliferating fraction was almost exclusively found among GFP<sup>hi</sup> cells, suggesting that Nur77-eGFP expression indeed marks a functionally distinct CD4 T cell population in the periphery of SKGNur mice, presumably driven, at least in part, by stronger recognition of self-pMHC in vivo and in vitro. Moreover, this functional heterogeneity is identifiable by the extent of the Nur77-eGFP reporter expression before frank disease onset.

Since both cytokine and self-pMHC–TCR interactions are essential for homeostatic T cell proliferation (37–39), we wanted to investigate whether differences in IL-7 responsiveness contributed to increased proliferation of GFP<sup>hi</sup> SKGNur CD4 T cells under steady-state conditions. Interestingly, GFP<sup>hi</sup> CD4 T cells are

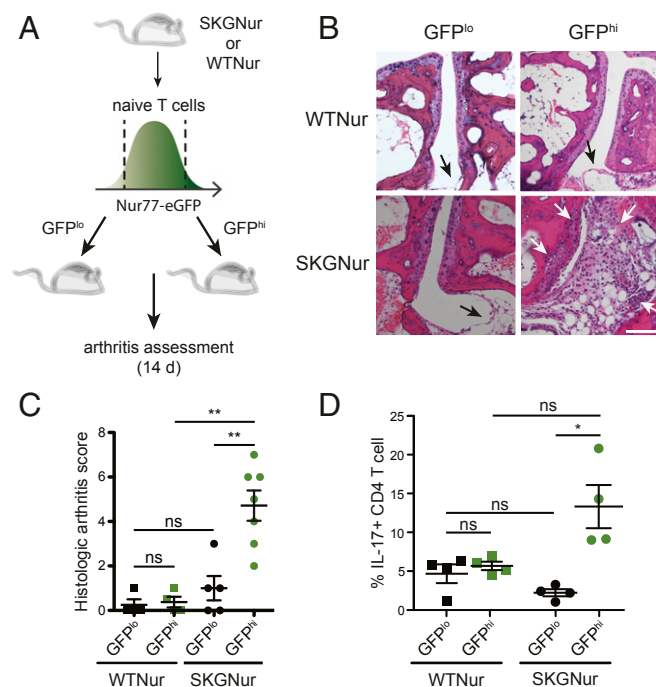
relatively insensitive to IL-7 signaling in comparison to GFP<sup>lo</sup> CD4 T cells from both SKGNur and control mice as assessed by phosphorylation of signaling transducer and activator of transcription 5 (STAT5) upon treatment with IL-7 (*SI Appendix, Fig. S44*). This result also correlated with lower levels of IL-7R $\alpha$  (CD127) on GFP<sup>hi</sup> compared with GFP<sup>lo</sup> CD4 T cells (*SI Appendix, Fig. S4B*). These data further support functional heterogeneity among the SKG T cell repertoire but do not account for increased proliferation of SKGNur GFP<sup>hi</sup> CD4 T cells at steady state, implying instead a role for antigen-dependent TCR signaling.

**Nur77-eGFP Expression in SKGNur CD4 T Cells Strongly Correlates with Their Arthritogenic Potential.** Since the Nur77-eGFP reporter appeared to mark more autoreactive T cells, we next asked whether this, in turn, correlated with their capacity to drive arthritis. We used an adoptive transfer model of arthritis (26, 40, 41) to determine whether reporter expression identified a more arthritogenic population of CD4 T cells. In this model, CD4 T cells transferred from SKG mice, but not from sera or CD8 or B cells, are sufficient to cause arthritis (26). As delineated in Fig. 2A, CD4<sup>+</sup>CD25<sup>−</sup> GFP<sup>hi</sup> and GFP<sup>lo</sup> naive T cells were sorted and adoptively transferred into severe combined immunodeficient (SCID) recipients. Initially, we found that, with sufficient time (>6 wk posttransfer), both GFP<sup>hi</sup> and GFP<sup>lo</sup> CD4 SKG T cells were capable of producing arthritis upon transfer to lymphopenic hosts, while WT T cells were not. This suggested that the entire

SKG T cell repertoire harbors T cells with pathogenic potential. Therefore, we next sought to evaluate whether a difference in onset and severity could be histologically detected. Indeed, transfer of CD4<sup>+</sup>CD25<sup>−</sup> naive T cells expressing the highest levels of GFP (GFP<sup>hi</sup>) resulted in an earlier onset (14 d after adoptive transfer) and more severe arthritis in recipient SCID mice than did the transfer of GFP<sup>lo</sup> naive CD4 T cells (Fig. 2B and C). This was characterized by increased synovial proliferation, inflammatory infiltrates, and early cartilage destruction (Fig. 2B, white arrows and C). This was not observed in SCID mice that received either SKGNur GFP<sup>lo</sup> CD4<sup>+</sup>CD25<sup>−</sup> naive T cells or WTNur GFP<sup>hi</sup> or GFP<sup>lo</sup> CD4<sup>+</sup>CD25<sup>−</sup> naive T cells, where the thin layer of synovial lining is maintained and infiltrating inflammatory cells are absent (Fig. 2B, black arrows and C). To assess whether these results were unique to the naive SKGNur GFP<sup>hi</sup> CD4 T cell population, the adoptive transfer experiment was repeated after sorting for GFP<sup>hi</sup> and GFP<sup>lo</sup> CD4<sup>+</sup>CD25<sup>−</sup> memory T cells (*SI Appendix, Fig. S5A*). We likewise found that the SKGNur GFP<sup>hi</sup> CD4<sup>+</sup>CD25<sup>−</sup> memory T cells were capable of producing a more severe and earlier onset of arthritis than SKGNur GFP<sup>lo</sup> T cells or BALB/c WTNur control T cells (*SI Appendix, Fig. S5B*). We conclude that arthritogenicity is a property of the entire SKG CD4 T cell repertoire but that T cells have varying arthritogenic potential, and, most importantly, that this arthritogenic potential can be identified by the amount of Nur77-eGFP expression and reflects their degree of self-reactivity.

Since the GFP<sup>hi</sup> population in SKGNur mice had greater arthritic potential, we asked whether the GFP<sup>hi</sup> population exhibited T cell effector functions distinct from those of the GFP<sup>lo</sup> population in SKGNur mice. To address this question, we determined their ability to generate IL-17 in vitro by stimulating sorted primary naive T cell populations with plate-bound anti-CD3 and CD28. Notably, we found that the percentages of IL-17-producing cells were 5- to 10-fold higher in the SKGNur GFP<sup>hi</sup> population than in the SKGNur GFP<sup>lo</sup> population. However, the difference in IL-17 production between GFP<sup>hi</sup> and GFP<sup>lo</sup> populations from WTNur mice was almost indistinguishable (*SI Appendix, Fig. S5C*). To determine whether this difference could be detected in our adoptive transfer model, transferred naive or memory CD4 T cells recovered from the recipient SCID mice were stimulated for 5 h ex vivo with phorbol myristate acetate (PMA) and ionomycin in the presence of brefeldin A to identify Th17 effector T cells capable of secreting IL-17. CD4 T cells recovered from SCID recipient mice that received SKGNur GFP<sup>hi</sup> naive or memory CD4 T cells had a significantly higher percentage of such IL-17-producing T cells than their GFP<sup>lo</sup> counterparts (Fig. 2D and *SI Appendix, Fig. S5D*). This increase in IL-17-producing cells was not observed in the CD4 T cells recovered from SCID mice that received WTNur GFP<sup>hi</sup> or GFP<sup>lo</sup> CD4 T cells. Collectively, our results suggest that the arthritogenic capacity of SKG CD4 T cells can be distinguished by their relative expression of Nur77-eGFP before disease onset, and corresponds to increased autoreactivity and proclivity to differentiate into pathogenic IL-17-secreting effectors in vitro and in vivo.

**Autoreactive Nur77-eGFP<sup>hi</sup> SKG T Cells Signal Poorly in Response to TCR Stimulation.** We next sought to understand why arthritogenic GFP<sup>hi</sup> SKG T cells preferentially differentiated into Th17 effectors. Since GFP levels reflect integrated TCR signaling, we hypothesized that differences in TCR signaling by this population of T cells may contribute to their Nur77-eGFP amounts (Fig. 3A). We already showed that GFP<sup>hi</sup> SKG T cells are more self-reactive, and therefore likely encounter more antigen in vivo (Fig. 1D and E). We next wanted to determine whether cell-intrinsic capacity to signal downstream of their TCRs differed between GFP<sup>hi</sup> and GFP<sup>lo</sup> T cells. To test this, we probed signaling events downstream of the TCR. By removing cells from in vivo antigen exposure and treating them with the polyclonal stimulus  $\alpha$ -CD3 in vitro, we were able to bypass any differences in TCR repertoire between the GFP<sup>hi</sup> and GFP<sup>lo</sup> CD4 T cell subsets. We first analyzed TCR-induced increases in cytoplasmic free calcium



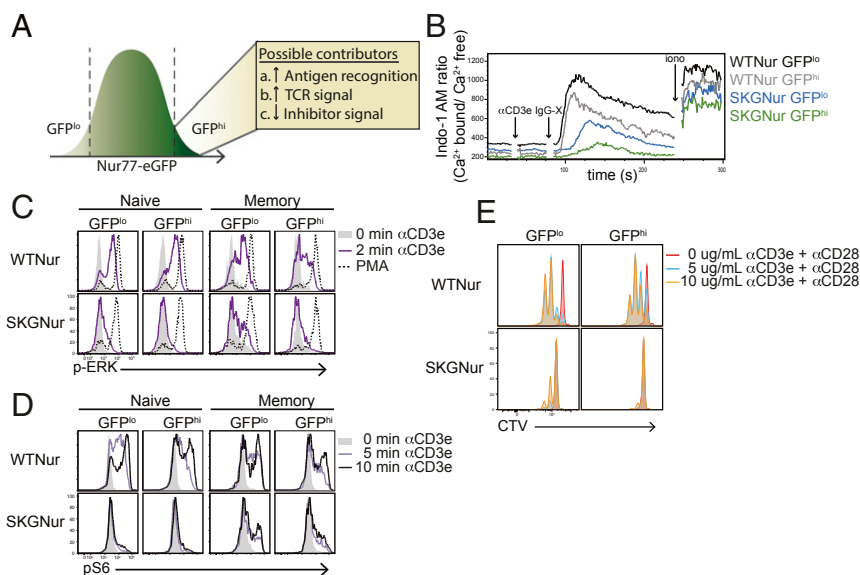
**Fig. 2.** Nur77-eGFP marks arthritogenicity of CD4 T cells in SKGNur mice. (A) The 10% highest and lowest Nur77-eGFP-purified CD4 T cells sorted on naive markers (CD62L<sup>hi</sup>CD44<sup>lo</sup>) and depleted of Treg marker CD25 were adoptively transferred into SCID recipients. To prevent complications of early-onset CD4 T cell inflammatory bowel disease after adoptive transfer of naive SKG T cells, WT Tregs were added back in a ratio of 1:3. (B) Joints were assessed by histological staining 14 d following adoptive transfer and scored in a blinded fashion. Black arrows indicate normal synovial lining, and white arrows indicate synovial proliferation and joint destruction. (Scale bar, 100  $\mu$ m.) (C) Dot plot of the histological score from SCID recipients that received GFP<sup>lo</sup> (black) or GFP<sup>hi</sup> (green) CD4 naive T cells from WTNur (square) or SKGNur (circle) mice. Each dot represents 1 biologically independent sample pooled from 3 experiments (mean  $\pm$  SEM). ns, not significant. (D) Dots represent percentage of IL-17-producing GFP<sup>+</sup> CD4 T cells from SCID recipient splenocytes stimulated in vitro with PMA and ionomycin for 5 h (mean  $\pm$  SEM).

in peripheral naive T cells. SKGNur CD4<sup>+</sup>CD25<sup>-</sup> T cells, irrespective of GFP expression, demonstrated profoundly dampened TCR-induced increases in cytoplasmic free calcium, consistent with the hypomorphic SKG Zap70 allele (Fig. 3*B* and *SI Appendix*, Fig. S14). The calcium responses of GFP<sup>hi</sup> SKGNur CD4 T cell responses were even more dampened than those of GFP<sup>lo</sup> CD4 T cells (Fig. 3*B*). Interestingly, an analogous trend was seen with GFP<sup>hi</sup> WTNur CD4 T cells relative to GFP<sup>lo</sup> WTNur CD4 T cells, suggesting that increased integrated TCR signaling input is associated with dampened downstream TCR signal transduction.

Analysis of TCR-induced extracellular signal-regulated kinase (ERK) phosphorylation in purified peripheral CD4 T cells similarly revealed that both GFP<sup>hi</sup> and GFP<sup>lo</sup> CD4<sup>+</sup>CD25<sup>-</sup> SKGNur T cells had a severe impairment in ERK phosphorylation after polyclonal TCR stimulation. This was observed in both the memory and naive peripheral CD4 T cell compartments (Fig. 3*C* and *SI Appendix*, Fig. S1*B*). By contrast, ERK activation in response to PMA, which bypasses TCR signaling, was normal, consistent with the well-defined role of Zap70 in regulating only proximal TCR signal transduction. In WTNur mice, GFP<sup>hi</sup> CD4 T cells appeared to have subtly decreased signaling capacity compared with GFP<sup>lo</sup> T cells, but the responses were still largely preserved, contrasting with the markedly impaired SKG responses (Fig. 3*C*). Similarly, GFP<sup>hi</sup>, more than GFP<sup>lo</sup>, CD4<sup>+</sup>CD25<sup>-</sup> SKGNur T cells had severely dampened phosphatidylinositol 3-kinase (PI3K)/Akt signaling downstream of polyclonal TCR stimulation as analyzed by S6 phosphorylation at Ser240/244 (Fig. 3*D*). Likewise, although WTNur GFP<sup>hi</sup> CD4 T cells exhibited a slightly dampened response compared with GFP<sup>lo</sup> CD4 T cells, PI3K/Akt signaling downstream of TCR stimulation was much more robust in WTNur cells when compared with SKGNur cells (Fig. 3*D*). Consistent with our calcium mobilization and phospho-flow studies, sorted GFP<sup>lo</sup> CD4<sup>+</sup>CD25<sup>-</sup> naive CD4 T cells from both SKGNur and WTNur mice proliferated more robustly to plate-bound  $\alpha$ CD3 stimulation than GFP<sup>hi</sup> naive CD4 T cells (Fig. 3*E*). Moreover, SKGNur CD4 T cells exhibited a clear proliferative defect in response to plate-bound  $\alpha$ CD3 stimulation as previously described (30), which stands in marked contrast to SKGNur GFP<sup>hi</sup> naive

CD4 T cells' ability to proliferate in response to self-antigen (Fig. 1*D* and *E*). Therefore, these results indicate that differences in GFP expression between the most and least arthritogenic SKGNur CD4 T cells are not attributable to signal transduction machinery but most likely reflect TCR autoreactivity to self-pMHC (antigen recognition), although the possibility of differential cytokine sensitivity contributing to proliferative differences cannot completely be excluded. It is possible that down-modulation of TCR signal strength in GFP<sup>hi</sup> relative to GFP<sup>lo</sup> CD4 T cells could represent a compensatory peripheral tolerance mechanism to prevent inappropriate activation of self-reactive clones. Collectively, these results suggest that differences in the measured TCR signal transduction events do not account for the unique propensity of GFP<sup>hi</sup> CD4 T cells from SKGNur mice to differentiate in vivo and in vitro into Th17 effectors.

**Up-Regulation of Several Inhibitory Receptors in SKG GFP<sup>hi</sup> Cells.** It was quite surprising that GFP<sup>hi</sup> CD4 T cells from SKGNur mice were able to exert their effector functions leading to joint immunopathology despite their profound impairment to in vitro TCR signal transduction (Fig. 3*B–D*). To account for the discrepancy between arthritogenic and signaling potential of the GFP<sup>hi</sup> cells as detected here, we next asked whether GFP<sup>hi</sup> CD4 T cells from SKGNur mice expressed much lower levels of inhibitory receptors, thereby permitting them to expand more rapidly in a lymphopenic environment, or whether they expressed higher levels of inhibitory receptors, further compromising their impaired TCR signaling capacity compared with GFP<sup>lo</sup> CD4 T cells. To address this, we examined the surface expression of several inhibitory receptors (PD-1, TIGIT, and CD73) that have been shown to dampen downstream TCR signaling, the last of which marks "anergic" T cell populations (42). GFP<sup>hi</sup> CD4 T cells expressed higher levels of these inhibitory receptors than their GFP<sup>lo</sup> counterparts, again suggesting that this may represent a compensatory tolerance mechanism to constrain self-reactive T cells (Fig. 4). However, higher expression levels of inhibitory receptors observed in the GFP<sup>hi</sup> population were not unique to SKGNur T cells since WTNur GFP<sup>hi</sup> CD4 T cells also had higher



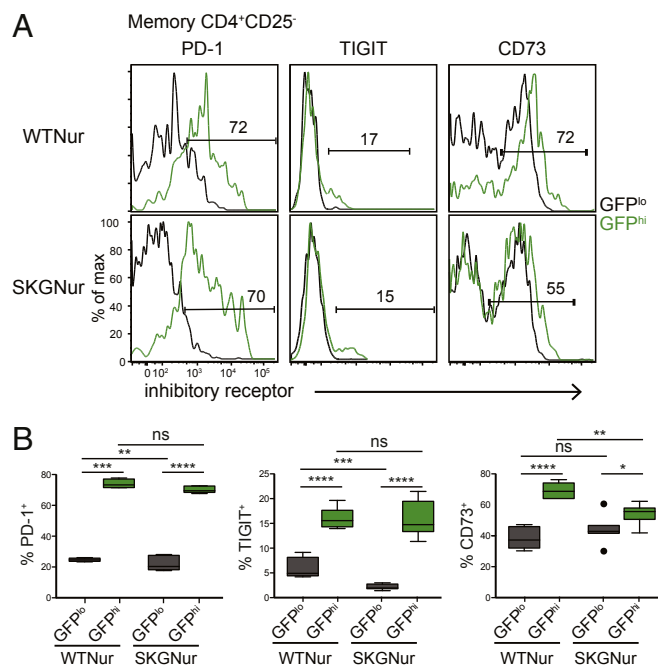
**Fig. 3.** Autoreactive Nur77-eGFP<sup>hi</sup> SKG T cells signal poorly. (A) Integrated TCR signaling inputs reflect Nur77-eGFP levels. (B) TCR-induced calcium increases in GFP<sup>lo</sup> and GFP<sup>hi</sup> negatively selected CD4<sup>+</sup>CD25<sup>-</sup> T cells were barcoded from WTNur and SKGNur mice. Data are representative of 4 to 5 mice in each group from 3 independent experiments. Indo-1 AM, fluorescent Ca<sup>2+</sup> indicator. Histograms represent phospho-ERK (p-ERK) (C) and p-S6 (D) levels analyzed in GFP<sup>lo</sup> and GFP<sup>hi</sup> CD4<sup>+</sup>CD25<sup>-</sup> T cells from WTNur and SKGNur mice by flow cytometry after stimulation with  $\alpha$ -CD3 $\epsilon$ . Data are representative of at least 8 mice in each group from 4 independent experiments. (E) Sorted CD4<sup>+</sup>CD25<sup>-</sup> naive GFP<sup>lo</sup> and GFP<sup>hi</sup> T cells were loaded with CTV dye and stimulated with plate-bound  $\alpha$ -CD3 $\epsilon$  and 2  $\mu$ g/mL  $\alpha$ -CD28 for 3 d. Cells were subsequently assessed for dye dilution by flow cytometry. Data are representative of 2 pooled mice in each group from 2 independent experiments.



levels of inhibitory receptors compared with WTNur GFP<sup>lo</sup> CD4 T cells. This is consistent with a recent report demonstrating that *NR4A* gene expression positively correlated with *PDCDI* (PD-1), *TIGIT*, and other inhibitory receptors in the setting of chronic antigen stimulation as part of a T cell-intrinsic program of hypo-responsiveness (43). In addition to CD73, folate receptor 4 (FR4)—another marker used to identify anergic T cells—was enriched in the GFP<sup>hi</sup> populations from WTNur and SKGNur mice (SI Appendix, Fig. S6A). Therefore, phenotypically anergic cells were compared between genotypes. While there did not appear to be a significant difference between the percentage of total anergic T cells between WTNur and SKGNur mice, the GFP<sup>hi</sup> T cells were clearly enriched for T cells with an anergic phenotype, albeit not as robustly in the SKGNur mice as in the WTNur mice (SI Appendix, Fig. S6B and C). Together, these results may account, in part, for the relative hypo-responsiveness we observed in GFP<sup>hi</sup> T cells (Fig. 3B–E). Therefore, it is all the more striking that SKGNur GFP<sup>hi</sup> CD4 T cells up-regulate inhibitory pathway receptors; appear to be enriched for phenotypically anergic cells; and yet, despite this, exhibit the capability to proliferate in response to autologous APCs, differentiate into IL-17-producing cells, and cause more severe arthritis.

**Enhanced IL-6 Response in GFP<sup>hi</sup> CD4 T Cells from SKGNur Mice.** Th17 differentiation depends not only on TCR signaling in response to pMHC but also on polarizing cytokines. Given the critical role of IL-6 in the development of arthritogenic Th17 cells in SKG mice (36), we examined in vitro responsiveness of SKG and WT CD4 T cells to IL-6. Phospho-flow cytometric analyses revealed that SKGNur GFP<sup>hi</sup> CD4 T cells have higher basal levels of phosphorylated-STAT3 (Y705) than GFP<sup>lo</sup> CD4 T cells (Fig. 5A and B). This difference was not observed among comparable populations from unstimulated WTNur CD4 T cells. Interestingly, inducible STAT3 phosphorylation in response to IL-6 stimulation in the GFP<sup>hi</sup> populations was more robust than that of the GFP<sup>lo</sup> populations, but this was observed in both SKGNur and WTNur mice. The overall response to IL-6 stimulation in both the SKGNur GFP<sup>hi</sup> and GFP<sup>lo</sup> CD4 T cells was much greater than in the comparable populations obtained from WTNur CD4 T cells (Fig. 5A and B). To investigate whether IL-6 hyperresponsiveness in SKG mice was due to differences in total STAT3 protein expression levels, we first compared the expression levels of STAT3 between WT and SKG mice. As shown in Fig. 5C, we found that STAT3 expression was comparable between WT and SKG CD4 T cells. To determine whether this phenomenon was isolated to IL-6 or whether it occurred with other cytokines that signaled through STAT3, we examined the effects of IL-27—an IL-6-related cytokine—on GFP<sup>hi</sup> and GFP<sup>lo</sup> CD4 T cells. GFP<sup>hi</sup> CD4 T cells were also more sensitive to IL-27 stimulation from both WTNur and SKGNur mice (SI Appendix, Fig. S7). However, IL-27 is unlikely to contribute to arthritis development in view of its role of limiting T cell-driven pathology, in part, by antagonizing the actions of IL-6 and restricting the expansion of Th17 cells (44, 45).

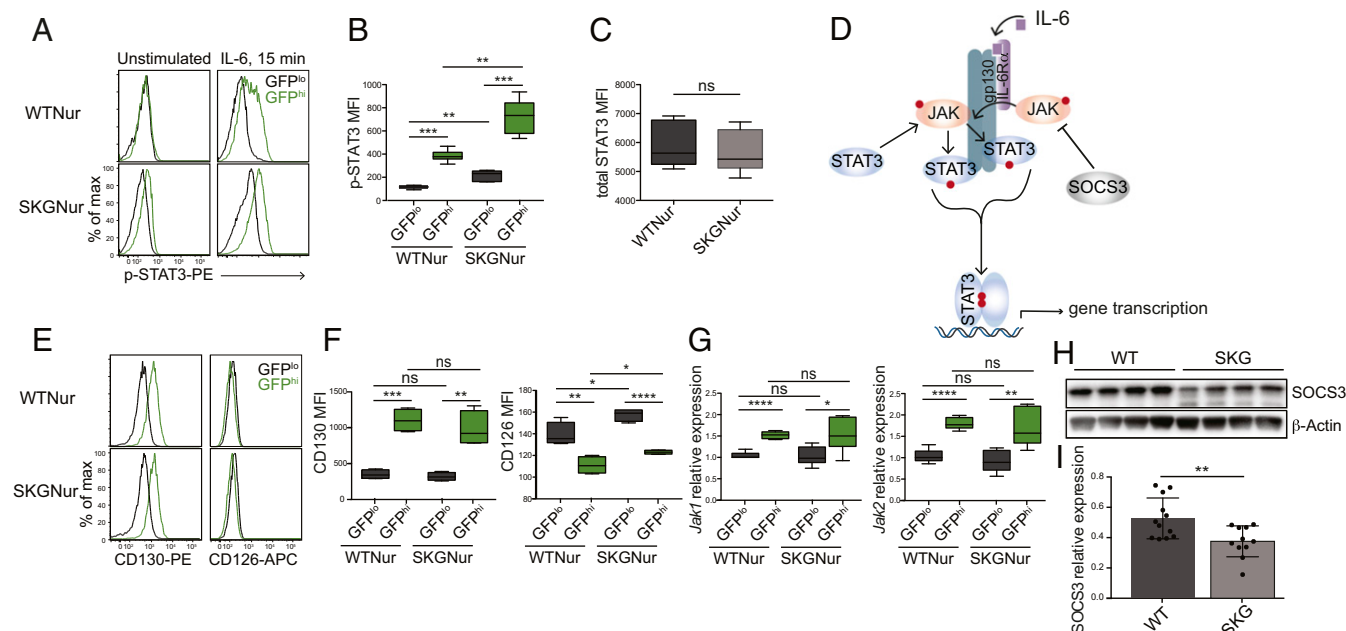
We next sought to examine the IL-6 pathway in greater detail (Fig. 5D). We first analyzed the expression of both components of the IL-6R, the ligand-binding IL-6R $\alpha$ -chain CD126 and the IL-6 family common receptor subunit gp130 (CD130), which is essential for signal transduction following cytokine engagement (46). As shown in Fig. 5E and F, the GFP<sup>hi</sup> populations expressed significantly higher amounts of CD130 than did the GFP<sup>lo</sup> populations. However, there was no significant difference in CD130 expression between WTNur and SKGNur mice. We did observe minimal differential expression of CD126 protein between the GFP<sup>hi</sup> and GFP<sup>lo</sup> populations, with lower amounts expressed in the GFP<sup>hi</sup> T cell subset (Fig. 5E and F). Although the impact of the small differences is not clear, it may represent shedding of IL-6R $\alpha$  in response to CD4<sup>+</sup> T cell activation (47, 48). These results indicate that the GFP<sup>hi</sup> and GFP<sup>lo</sup> populations differ in their expression of CD130, perhaps contributing to relatively increased IL-6 responses by GFP<sup>hi</sup> T cells.



**Fig. 4.** Inhibitory receptors are up-regulated in GFP<sup>hi</sup> CD4 T cells. (A) Expression levels of inhibitory receptors assessed by flow cytometry in memory CD4<sup>+</sup>CD25<sup>-</sup> cells of GFP<sup>lo</sup> and GFP<sup>hi</sup> populations from WTNur or SKGNur mice. Results shown are representative of 2 independent experiments. % of max, percentage of maximum. (B) Quantification of percentage of positive inhibitory receptor levels by 2-tailed Student's *t* test. Box plots describe the median and interquartile range (IQR); whiskers extend to the largest value, but no further than 1.5 \* IQR; and data points beyond whiskers are outlying points. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001. ns, not significant.

Since these differences did not seem to account for the greater IL-6 response in SKGNur mice, we further assessed key signaling molecules downstream of the IL-6 signaling pathway, such as *Jak1*, *Jak2*, and *SOCS3*. Similar to surface expression of CD130, GFP<sup>hi</sup> CD4 T cells expressed higher amounts of *Jak1* and *Jak2* messenger RNA than did GFP<sup>lo</sup> CD4 T cells (Fig. 5G). Nevertheless, the expression levels of *Jak1* and *Jak2* transcripts were comparable between the respective CD4 T cell populations isolated from GFP populations in WTNur and SKGNur mice (Fig. 5G). Thus, increased expression of CD130, *Jak1*, and *Jak2* in GFP<sup>hi</sup> CD4 T cells might contribute to enhanced IL-6 responses in this population, but both WTNur and SKGNur shared these properties. Immunoblot analyses indicated that *SOCS3* protein, a key negative regulator of IL-6 signaling (49), was down-regulated in SKGNur CD4 T cells (Fig. 5H and I). Our results suggest that the hyperresponsiveness to IL-6 observed in autoreactive SKGNur CD4 T cells exposed to chronic antigen stimulation may be due, at least in part, to decreased *SOCS3*-dependent suppression. We propose that SKGNur GFP<sup>hi</sup> CD4 T cells exhibit uniquely robust IL-6 responses because they both up-regulate the IL-6 proximal signaling machinery and coordinately express lower levels of the negative regulator *SOCS3*. Other regulators of this pathway could also influence the differential IL-6R responses of T cells from the WT vs. SKG genotypes.

**Nur77 Identifies Increased Frequencies of Antigen-Stimulated CD4 T Cells in Arthritic SKG Synovium.** A central challenge in isolating bona fide antigen-activated T cells from inflamed arthritic joints in patients with RA is differentiating these from T cells activated via cytokines in the inflammatory milieu (50). Recently, we developed technologies to detect induction of endogenous Nur77 protein following TCR and B cell receptor stimulation in mouse and human T cells (21, 22, 24). Since Nur77 is induced downstream of TCR but



**Fig. 5.** Enhanced IL-6 response in GFP<sup>hi</sup> CD4 T cells from SKGNur mice. (A) Levels of phospho-STAT3 (p-STAT) (pY705) in GFP<sup>lo</sup> and GFP<sup>hi</sup> total CD4<sup>+</sup>CD25<sup>-</sup> T cell populations from WTNur and SKGNur mice  $\pm$  IL-6 treatment for 15 min. % of max, percentage of maximum. (B) Quantification of p-STAT3 mean fluorescence intensity (MFI) upon IL-6 stimulation from A. Data are pooled from 2 independent experiments ( $n = 6$  mice per group).  $^{**}P < 0.01$ ,  $^{***}P < 0.001$ . (C) Quantification of total STAT3 MFI in WTNur ( $n = 5$ ) and SKGNur ( $n = 10$ ) total CD4<sup>+</sup>CD25<sup>-</sup> T cells. ns, not significant. (D) IL-6 signaling pathway. (E) Surface expression of CD130 and CD126 in GFP<sup>lo</sup> and GFP<sup>hi</sup> CD4<sup>+</sup>CD25<sup>-</sup> T cells. PE, phycoerythrin. (F) Quantification of CD130 and CD126 MFI ( $n = 4$  per group from 2 independent experiments).  $^{*}P < 0.05$ ,  $^{**}P < 0.01$ ,  $^{***}P < 0.001$ ,  $^{****}P < 0.0001$ . (G) Real-time RT-PCR measurements of *Jak1* and *Jak2* messenger RNA levels in sorted GFP<sup>lo</sup> and GFP<sup>hi</sup> CD4 T cells from WTNur and SKGNur mice ( $n = 5$  to 7 mice per group from 2 independent sorts).  $^{*}P < 0.01$ ,  $^{***}P < 0.001$ ,  $^{****}P < 0.0001$ . In B, F, and G, all box plots represent quantification of MFI or RNA relative expression (center line, median; box limits, upper and lower quartiles; whiskers, 1.5 $\times$  interquartile range). (H) Immunoblot analysis of SOCS3 and  $\beta$ -actin in unstimulated, negatively selected CD4 T cells from SKG and WT mice. Data are representative of 3 experiments. (I) Quantification of SOCS3 protein levels normalized to  $\beta$ -actin pooled from 3 immunoblot experiments as shown in H, with 11 to 12 mice in each group.  $^{**}P < 0.01$ .

not cytokine receptor signaling (Fig. 6A), and we observed increased expression of Nur77-eGFP in arthritic joints of SKG mice (Fig. 1A), we determined whether endogenous Nur77 protein was also up-regulated in T cells after arthritis induction by zymosan challenge in SKG mice. Indeed, when examining the CD4 T cell population infiltrating arthritic SKG joints after zymosan treatment, we found up-regulation of endogenous Nur77 protein in a subset of CD4 T cells from SKG arthritic joints compared with nonarthritic phosphate-buffered saline (PBS)-treated SKG mice (Fig. 6B and gating in *SI Appendix*, Fig. S1D). Consistent with intracellular Nur77 expression, Nur77 transcript levels (*Nr4a1*) were increased  $\sim 6$ -fold in the population of joint-infiltrating CD4 T cells from zymosan-treated SKG mice (Fig. 6C).

The discrepancy between the greater percentage of CD4 T cells expressing high GFP amounts (Fig. 1A) compared with endogenous Nur77 may be due to the long in vivo half-life of GFP in comparison to endogenous Nur77 (22), but it also suggests a high degree of heterogeneity in the autoreactivity of the CD4 T cell population within the arthritic joint. Together, these data show that zymosan challenge in SKGNur mice, which triggers activation of autoreactive CD4 T cells, led to up-regulation of a population of cells that expressed higher levels of endogenous Nur77 expression in the inflamed joints. Since Nur77 marks antigen-dependent TCR signaling specifically, and the half-life of endogenous Nur77 is short (51), these results suggest that a subset of the joint-infiltrating CD4 T cells in SKG mice are recognizing an intraarticular antigen(s).

**Nur77 Protein, a Specific Indicator of TCR Signaling, Identifies Increased Frequencies of Antigen-Stimulated CD4 T Cells in Human RA Synovium.** Utilizing methodology we optimized for intracellular Nur77 staining in human lymphocytes (21), we used endogenous Nur77 protein expression to determine whether

joint-infiltrating CD4 T cells in human RA patients might be recognizing an intraarticular antigen, and as proof of concept to establish whether this marker could, in turn, be subsequently used to identify arthritogenic TCR specificities in RA patients. We analyzed Nur77 expression in synovial T cells from 8 patients undergoing a surgical joint procedure who met the American College of Rheumatology classification criteria for seropositive (rheumatoid factor or anti-CCP antibody-positive) RA (*SI Appendix*, Table S1). We found a significant increase in a fraction of Nur77<sup>+</sup> CD4 T cells isolated from synovial tissue from patients with seropositive RA compared with matched peripheral blood mononuclear cells (PBMCs) from the same donor (Fig. 6D and E and human gating in *SI Appendix*, Fig. S8), suggesting an enrichment of antigen-reactive cells in the joint. Nur77 protein expression in CD4 T cells obtained from the RA joints far exceeded that isolated from synovial tissue of patients with a diagnosis of osteoarthritis, a nonautoimmune degenerative form of arthritis (Fig. 6D and F and *SI Appendix*, Fig. S8). Importantly, the enrichment of endogenous Nur77 was only observed in synovial infiltrating CD4 T cells and not in CD8 T cells in RA synovium (Fig. 6D), consistent with the genetic link to specific MHC class II alleles and the role of CD4 T cells in the pathogenesis of RA (3–6, 8). These results support the hypothesis that pathogenic CD4 T cells are enriched in RA joints and are likely recognizing an intraarticular autoantigen(s).

**SOCS3 Transcripts Are Down-Regulated in RA Synovial Tissue.** Similar to the SKG model, the pleiotropic cytokine IL-6 is thought to contribute to the differentiation of Th17 cells in human RA, and targeting the IL-6R with clinically used humanized monoclonal antibodies leads to RA disease improvement (52, 53). Because SOCS3 induction is critical to prevent prolonged and enhanced IL-6 signaling in vivo (54), we wanted to investigate whether SOCS3 levels may be inappropriately reduced in patients with



RA, creating a permissive environment for IL-6 signaling as seen in the SKG mouse model of arthritis. Indeed, Ye et al. (55) found that peripheral CD4 T cells from patients with RA were associated with an aberrant STAT3 signaling network and had, in fact, down-regulated *SOCS3* expression. We then searched general public repository databases for available transcriptome data from RA synovial tissue. Thirteen studies met our inclusion criteria, comprising over 300 bulk synovial samples (*SI Appendix, Table S2*). Leveraging all collected publicly available microarray data, we specifically looked into the *SOCS3* gene. We found that *SOCS3* was down-regulated in RA synovial tissue compared with healthy controls with a fold change of 0.61 and *P* value of  $5E-11$  (Fig. 7 *A* and *B*). Distribution plots per individual study are shown in *SI Appendix, Fig. S9*. Furthermore, we interrogated the expression of additional IL-6 signaling pathway components and found minimal to no differences between patients with RA and controls, apart from *JAK2* (Fig. 7 *B* and *C*). These results suggest a mechanism whereby IL-6 signaling may go unchecked and contribute to persistent inflammatory responses in the RA joint. Some of these differences may relate to differences in cell type and inflammatory state between RA and healthy control synovium. Studies are underway to investigate whether infiltrating CD4 T cells from RA synovium contribute to this phenotype.

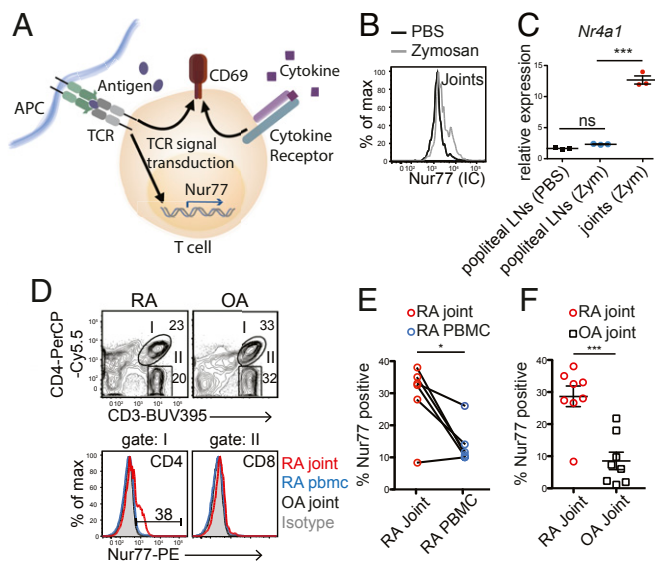
## Discussion

Here, we describe an innovative approach, through the detection and monitoring of Nur77 expression, to distinguish putative autoreactive T cells with increased arthritogenic potential from T cells activated by cytokine signaling. Indeed, we find an enrichment of intracellular Nur77<sup>+</sup> CD4 T cells in both human RA and murine autoimmune arthritis. Although RA synovial tissue has recently been phenotyped with attention placed on infiltrating “peripheral helper” T cells, little data exist on infiltrating T cell antigen reactivity or specificity (56–61). The ability to identify Nur77<sup>+</sup> CD4 T cells from RA synovial tissue obtained from patients with well-established and, in most cases, advanced “end-stage” joint disease suggests that antigen-specific CD4 T cell responses continue to persist in diseased joints. While we were able to exclude Tregs among the arthritogenic Nur77-GFP<sup>hi</sup> cells in the joints of SKG mice, we could not do so when endogenous Nur77 staining was used in patients. A subset of these synovial Nur77<sup>+</sup> CD4 T cells may include Tregs, although Tregs make up only ~10% of RA joint-infiltrating CD4 T cells (62). Our future studies will further characterize the synovial CD4 T cell subsets that express high levels of Nur77 (*Nr4a1*) using single-cell RNA sequencing. For clinical investigators, these results highlight the importance of studying the relevant tissues, rather than blood samples, to elucidate antigen-driven events in relevant target tissues.

The ability to use Nur77 to track antigen-activated T cells influenced our decision to take advantage of the Nur77-eGFP reporter to explore functional heterogeneity of the CD4 T cell repertoire before and after frank autoimmune disease onset in the SKG mouse model of arthritis. The SKGNur reporter revealed the following: (1) SKG thymocytes are able to up-regulate a reporter of TCR signaling during thymic positive selection despite their severely impaired TCR signaling capability, presumably due to high-affinity self-pMHC TCRs being selected; (2) further TCR stimulation occurs in the periphery (due to chronic autoantigen stimulation, further repertoire pruning, or both); and (3) highly autoreactive, arthritogenic T cells, despite (or rather because of) profound deficiency in their TCR signal transduction capacity, are hyperresponsive to IL-6 stimulation due, at least in part, to up-regulation of IL-6 signaling machinery and decreased *SOCS3* expression. This provides a mechanism whereby an autoreactive repertoire with impaired TCR signaling, as seen in the SKG model, is able to break tolerance, differentiate more readily into pathological Th17 effector cells in the presence of IL-6, and cause arthritis.

That SKG T cells with severely impaired signaling can effectively differentiate into Th17 effector cells that mediate an inflammatory, destructive arthritis akin to RA seems counterintuitive. Yet, there are multiple reports linking immune deficiency with immune dysregulation and autoimmunity (6, 26, 29, 30, 42, 63–65). It has been suggested that the partial reduction in the number or function of T cells can disturb a “tolerogenic balance,” and thereby generate a combination of immunodeficiency and hyperimmune dysregulation (65). The precise mechanism remains elusive, although murine models suggest multiple factors are at play, including selection of an autoimmune TCR repertoire, lymphopenia-induced exaggerated homeostatic proliferation precipitating autoimmune disease, deficient Treg function, resistance to Treg suppression, and T cell dysregulation causing failed induction of anergy in autoreactive T cells despite chronic antigen stimulation (42, 63). In addition to the SKG mouse model, the *Zap70* allelic series and LAT mutated mice reveal a causal link between impaired TCR signaling associated with immune dysregulation and autoimmunity (35, 66–69). Similarly, mouse models with cytokine signaling defects, such as in *STAT5A/5B*-deficient mice, can also serve as examples of immunodeficiency and immune dysregulation and suggest a synergistic role for cytokine signaling in the activation of self-reactive T cells (65, 70).

The link between immunodeficiency and autoimmunity has long been appreciated in humans. Perhaps it is most evident in patients with primary immunodeficiency, where up to 20 to 25%



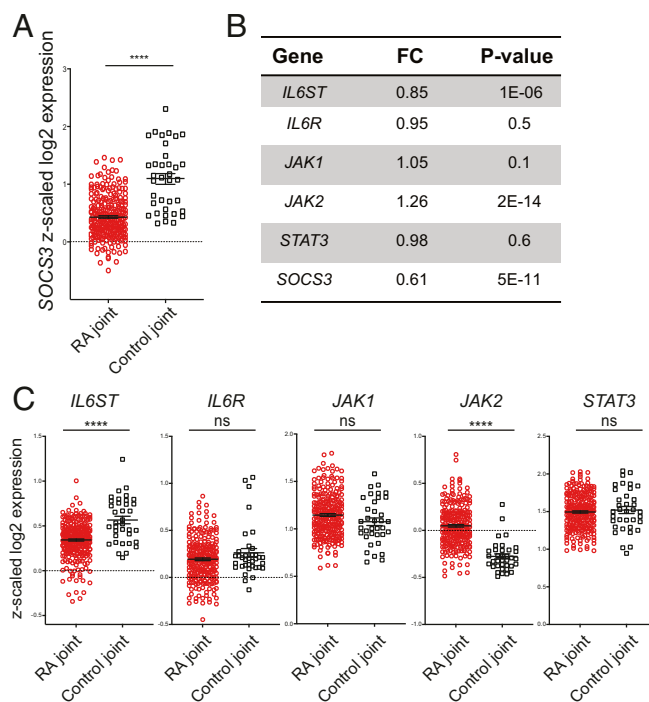
**Fig. 6.** Reporter of TCR signaling can be used to identify antigen-activated CD4 T cells in human RA synovium. (A) Depiction of Nur77 induction by TCR signal transduction, but not cytokine signaling. (B) Histogram shows the overlay of endogenous Nur77 protein levels in the infiltrating CD4<sup>+</sup>CD25<sup>+</sup> T cells from joints of SKGNur mice treated with phosphate-buffered saline (PBS) or zymosan. Data are representative of >10 mice in at least 5 independent experiments. (C) Comparison of messenger RNA transcript levels of *Nr4a1* (Nur77) in sorted CD4<sup>+</sup>CD25<sup>+</sup> T cells from lymph nodes (LNs) or joints of SKGNur mice treated with either PBS or zymosan (Zym). Each dot represents a biologically independent sample pooled from 2 independent experiments with 3 mice per group (mean  $\pm$  SEM), using repeated measures ANOVA. \*\*\**P*  $\leq$  0.001. ns, not significant. (D, Top) Flow cytometry plots of RA and osteoarthritis (OA) synovial tissue T cells demonstrate T cell populations from LiveCD45<sup>+</sup> RA and OA synovium. PerCP-Cy5.5, Peridinin-Chlorophyll-protein Cyanine5.5. (D, Bottom) Histograms represent Nur77 expression in CD4 (gate I) and CD8 (gate II) T cells from synovial RA (red) and OA (green) tissue, RA PBMCs (blue), and isotype control (filled-in gray histogram). PE, phycoerythrin; % of max, percentage of maximum. Dot plots demonstrate percentage of Nur77<sup>+</sup> CD4 T cells (gate I) from paired RA PBMCs and joints (*n* = 6) (E) and percentage of Nur77<sup>+</sup> CD4 T cells from 8 RA and 8 OA synovial tissues (F). \**P*  $\leq$  0.05 and \*\*\**P*  $\leq$  0.001 by 2-tailed Student's *t* test. Each dot represents a biologically independent sample (mean  $\pm$  SEM).

may develop autoimmune cytopenias, RA, or other autoimmune diseases (71–73). The greatest risk has been associated with partial T cell immunodeficiencies and common variable immunodeficiency (73). The reverse association has also been described. In the setting of primary autoimmunity, despite the presence of activated immune cells mediating disease, observational studies have found an increased susceptibility to serious infections (independent of immune-suppressive medications) and premature aging of the immune system, suggesting that impaired immune function might be a primary defect that, in turn, subverts tolerance (74–77). For example, RA CD4 T cells have the paradoxical ability to differentiate into pathogenic effector cells despite their impaired response to TCR engagement (6, 7, 11–18). Importantly, this paradox is observed in the SKG model of arthritis, making it one of the few mouse models to capture the contribution of an autoreactive TCR repertoire with impaired TCR signal transduction to the pathogenesis of arthritis.

Notably, our results uncover an unexpected mechanism for initiation of autoimmune arthritis involving cross-talk in peripheral T cells between signaling via the TCR and responsiveness to cytokine-dependent cues through IL-6R. We show that chronic TCR stimulation of GFP<sup>hi</sup> T cells not only results in compensatory changes in TCR signal transduction and inhibitory coreceptor expression but also heightened sensitivity to IL-6 that is due, at least in part, to increased expression of IL-6 signaling machinery, as well as decreased expression of SOCS3. Since IL-6 signaling drives the differentiation of CD4 T cells into arthritogenic Th17 effector cells and its presence is indispensable for arthritis development in SKG mice, our observations provide a mechanistic link between T cell self-reactivity and pathogenicity (36). While IL-6 is critically important for the differentiation of CD4 T cells into IL-17-secreting CD4 Th cells, it is also possible that IL-6R signaling integrates with TCR signaling to promote survival of autoreactive T cells in the SKG mouse. Limited literature exists on the integration of TCR and IL-6 cytokine signaling in this capacity. Their coincident responses may create a feed-forward loop whereby the presence of IL-6 protects CD4 T cells from activation-induced cell death, promotes their expansion and survival in the periphery, and participates in an IL-17A/IL-6-positive feedback loop promoting autoimmunity (78, 79). Further efforts delineating the networks involved in TCR and cytokine signaling integration in the setting of chronic (auto)antigen stimulation are underway.

SOCS3 may provide an additional link between IL-6 and TCR signaling networks (80, 81). SOCS3 is the most abundant of the SOCS family member expressed in unstimulated naive T cells. Its overexpression appears to inhibit TCR signaling and Th cell proliferation (80, 82), whereas decreased SOCS3 activity has been implicated in murine autoimmune arthritis (81–83) and in human RA (55, 83–85). Furthermore, the suppression of SOCS3 by antigen stimulation of naive CD4 T cells has been reported following acute TCR stimulation (80, 82). In this study, we propose that autoantigen-dependent stimulation of the skewed TCR repertoire in SKG mice may lead to the observed SOCS3 suppression in their CD4 T cells. Suppression of SOCS3, possibly driven by chronic autoantigen-dependent TCR signaling, may also result in altered IL-6 and TCR regulation, further promoting the arthritogenic potential of GFP<sup>hi</sup> cells in the SKGNur mice. Additionally, we identified reports of gene expression in RA synovium and found a highly significant reduction of *SOCS3*, suggesting the aforementioned mechanism may be relevant in RA and related autoimmune diseases. These results will need to be validated in CD4 synovial T cells from patients with RA. Further work delineating the regulation of SOCS3 in the setting of chronic (auto)antigen stimulation remains to be determined.

Thus, our study highlights the functional importance of identifying and studying the arthritogenic T cell population in SKG mice both before and after frank disease initiation, with translatable implications for human RA. Further dissection of the altered TCR and cytokine signaling networks in arthritogenic human CD4 T cells may expand our understanding of RA pathogenesis and, in so doing, may reveal targets for novel therapeutic



**Fig. 7.** Comparison of gene expression levels in synovium from patients with RA. The gene expression values were log<sub>2</sub>-transformed and z-scaled. The gene expression levels are summarized in the form of mean ± SEM. (A) Synovial *SOCS3* expression levels. (B) Table with summary statistics for genes in the IL-6 signaling pathway. FC, fold change. (C) Expression levels for *IL6ST*, *IL6R*, *JAK1*, *JAK2*, and *STAT3* genes. *P* values were computed by performing the nonparametric 2-sample Mann–Whitney–Wilcoxon test. \*\*\*\**P* < 0.0001. ns, not significant.

approaches to RA. In addition, our study lays the groundwork for the identification of arthritogenic T cell clones and their receptors, as well as bona fide autoantigens not only in the SKG model but also in human RA. Our work also provides a platform for investigators to identify autoantigen-specific T cells in other immunologically mediated human diseases in which the inciting antigen is not known—autoimmunity, cancer, and checkpoint blockade, as well as transplant rejection.

## Methods

**Data Reporting.** No statistical methods were used to predetermine sample size. The human studies were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment.

**Mice.** BALB/c and SCID BALB/c mice were purchased from The Jackson Laboratory, and SKG and Nur77-eGFP Tg mice were kindly provided by Shimon Sakaguchi, Kyoto University, Kyoto, Japan, and Julie Zikherman, University of California, San Francisco (UCSF), respectively. Nur77-eGFP mice were backcrossed to BALB/c more than 8 times and crossed to SKG mice to make SKGNur mice. All mice were housed and bred in specific pathogen-free conditions in the Animal Barrier Facility at UCSF according to the University Animal Care Committee and NIH guidelines. All animal experiments were approved by the UCSF Institutional Animal Care and Use Committee.

**In Vivo Treatments.** Zymosan A (Sigma–Aldrich) suspended in saline at 10 mg/mL was kept in boiling water for 10 min. Zymosan A solution (2 mg) or saline was intraperitoneally injected into 8- to 12-wk-old mice. Adoptive transfer experiments were performed as described (26, 40). The highest and lowest 5% Nur77-eGFP-purified CD4 T cells sorted on naive (CD62L<sup>hi</sup>CD44<sup>lo</sup>) or memory (CD62L<sup>lo</sup>CD44<sup>hi</sup>) markers and depleted of the Treg marker CD25 were adoptively transferred by tail vein injection into SCID recipients. To prevent complications of weight loss and early onset of inflammatory bowel disease after adoptive transfer of naive SKG T cells, WT Tregs were added back in a ratio of 1:3.

**Histology and Histopathology Scoring Criteria.** Ankle joints were fixed in buffered 10% formalin, and paraffin-embedded sections were decalcified and stained with hematoxylin and eosin by the UCSF mouse pathology core. Ankle joints were scored in a blinded fashion using a modified protocol (86). Further details are provided in *SI Appendix*. A sum total (all joints) animal score was determined. Parameters for the various groups were then compared using Student's *t* test.

**Murine Synovial Tissue Preparation.** Synovial tissues from ankle joints were digested with 1 mg/mL Collagenase IV (Worthington) in RPMI 1640 medium for 1 h at 37 °C; digested cells were filtrated through a nylon mesh to prepare single-cell suspensions.

**Complementary DNA Synthesis and qRT-PCR.** Total RNA was purified using TRIzol (Invitrogen) or the RNeasy Micro Kit (QIAGEN). Complementary DNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. TaqMan real-time PCR was performed using TaqMan Real-Time PCR Master Mix (Invitrogen), and TaqMan primer and probe were purchased from IDT. The relative abundance of all target genes was normalized to the abundance of an internal reference gene. Primer sequences are provided in *SI Appendix*.

**AMLR.** The 10% highest and lowest Nur77-eGFP-sorted CD4<sup>+</sup>CD25<sup>−</sup>CD62L<sup>hi</sup>CD44<sup>lo</sup> naive T cells from SKGNur and WTNur lymph node and spleen were labeled with 5  $\mu$ M CTV according to the manufacturer's instructions (Life Technologies) before culturing in a 1:10 ratio with  $5 \times 10^5$  nonirradiated BALB/c splenic APCs (prepared via Thy1.2<sup>+</sup> cell depletion by MACS; Miltenyi Biotec) in a 96-well, round-bottom plate in complete RPMI medium for 5 d. Cells were harvested and stained with fixable Live/Dead stain, anti-CD4, and anti-TCR- $\beta$ , and were analyzed by flow cytometry. A FlowJo Proliferation Platform was used to determine the percent divided.

**Intracellular Nur77 Staining.** Nur77 staining assays in murine and human cells were performed as previously described (21). All other intracellular staining methods can be found in *SI Appendix*.

**Human Research.** Research involving human subjects was performed according to the UCSF Institutional Review Board (IRB) through an approved protocol (IRB no. 13-11485) with appropriate informed consent as required. Patients with seropositive RA fulfilled the American College of Rheumatology 2010 RA classification criteria.

**Patient Identification for Synovial Tissue.** Through use of the UCSF Clinical Data Research Consultations Office, patients with seropositive RA referred to UCSF orthopedic surgery and scheduled for joint replacement, synovial biopsy, or synovectomy were identified through data extraction of the UCSF Medical Center's electronic medical records and recruited to the study. A

discussion of patient inclusion/exclusion criteria and synovial tissue disaggregation is provided in *SI Appendix*.

**Human PBMC Preparation.** Whole human blood was collected from patients with RA undergoing joint surgery in BD Vacutainer tubes (Becton Dickinson) lined with sodium heparin and gently mixed. Monocytes were isolated by density gradient Ficoll separation as previously described (21).

**Genomic Data Collection and Preprocessing.** A comprehensive search for publicly available microarray data at the National Center for Biotechnology Information Gene Expression Omnibus (87) database (<https://www.ncbi.nlm.nih.gov/geo/>) was performed. The keywords "rheumatoid arthritis," "synovium," "synovial," and "biopsy" among organism "*Homo sapiens*" and study type "Expression profiling by array" were used. The criteria for the dataset exclusion from the search results were the lack of annotations and low probe platforms. We were able to collect 13 datasets with 312 synovial biopsies (*SI Appendix, Table S2*). Among them, there were 276 samples with an RA diagnosis confirmed in later follow-ups and 36 healthy tissue biopsies obtained from joint or trauma surgery. The gene expression measurements were made on 4 platforms from the manufacturers Affymetrix and Illumina and using 2 common noncommercial chips. A discussion of raw data preprocessing is provided in *SI Appendix*.

**Statistics.** Results from independent experiments were pooled whenever possible; all data were analyzed by comparison of means using paired or unpaired 2-tailed Student's *t* tests using Prism version 5.0f for Mac (Graph-Pad Software). Data in all figures represent mean  $\pm$  SEM unless otherwise indicated. Differences were considered significant at  $P < 0.05$ : \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ .

Additional details on materials and methods are provided in *SI Appendix*, including antibodies used, flow cytometry, intracellular staining methods, calcium mobilization, in vitro proliferation assay, and immunoblot analysis.

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